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The Expanded Octarepeat Domain Selectively Binds Prions and Disrupts Homomeric Prion Protein Interactions*

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Insertion of additional octarepeats into the prion protein gene has been genetically linked to familial Creutzfeldt Jakob disease and hence to *de novo* generation of infectious prions. The pivotal event during prion formation is the conversion of the normal prion protein (PrP^{C}) into the pathogenic conformer PrP^{Sc}, which subsequently induces further conversion in an autocatalytic manner. Apparently, an expanded octarepeat domain directs folding of PrP toward the PrP^{Sc} conformation and initiates a self-replicating conversion process. Here, based on three main observations, we have provided a model on how altered molecular interactions between wild-type and mutant PrP set the stage for familial Creutzfeldt Jakob disease with octarepeat insertions. First, we showed that wild-type octarepeat domains interact in a copper-dependent and reversible manner, a "copper switch." This interaction becomes irreversible upon domain expansion, possibly reflecting a loss of function. Second, expanded octarepeat domains of increasing length gradually form homogenous globular multimers of 11–21 nm in the absence of copper ions when expressed as soluble glutathione *S*-transferase fusion proteins. Third, octarepeat domain expansion causes a gain of function with at least 10 repeats selectively binding PrP^{Sc} in a denaturant-resistant complex in the absence of copper ions. Thus, the combination of both a loss and gain of function profoundly influences homomeric interaction behavior of PrP with an expanded octarepeat domain. A multimeric cluster of prion proteins carrying expanded octarepeat domains may therefore capture and incorporate spontaneously arising short-lived PrP^{Sc}-like conformers, thereby providing a matrix for their conversion.

Prion diseases are transmissible neurodegenerative diseases that, uniquely, in humans can be of genetic, sporadic, or infectious origin. Cases of the most prevalent human prion disease, Creutzfeldt Jakob disease (CJD),[2] are ~15% genetic, 85% sporadic, and only <1% linked to infection. In genetic or familial CJD (fCJD), germ line mutations in the prion protein gene (*PRNP*) initiate a neurodegenerative disease that subsequently becomes transmissible (1, 2). This phenomenon has not been reported for other mammalian prion diseases that are more prevalent and seem to have mostly an infectious origin (1, 3). Major animal prion diseases include scrapie of sheep and goats, bovine spongiform encephalopathy of cattle, and chronic wasting disease of American mule deer and elk. Transmissibility between species is limited and regulated by a species barrier that is determined by genetic differences in the *PRNP* gene and eventually by other genes (4, 5). In contrast to the seemingly exclusive occurrence of genetic prion disease in humans, polymorphisms in *PRNP* are known to occur in many species and to influence prion infection susceptibility (3).

The essential molecular component of prions is PrP^{Sc}, a pathological conformer of the prion protein that replicates without the need for nucleic acids (1). Once initiated, the prion replication mechanism is characterized by the conformational conversion of the cellular ("normal") isoform of the prion protein (PrP^{C}) into PrP^{Sc}, which in turn induces further conversion of PrP^{C}, thus propagating the PrP^{Sc} conformation (1). Currently, 55 pathogenic mutations have been identified that cause inherited CJD in humans. Of those, 24 are missense mutations and 27 are insertion mutations consisting of up to 9 additional 24-bp repeats and corresponding to an increase ("expansion") in the number of octarepeats, of which there are normally four consecutive copies (3). Interestingly, the clinical phenotype of fCJD with insertional mutations can mimic that of Huntington disease in the early phases of the disease (6).

Attempts at rebuilding genetic mutations that cause fCJD in cell or animal models in order to reproduce *de novo* prion genesis have not been successful so far (7), suggesting that either unknown factors in the human genetic background or lifespan contribute to genetic prion formation. Prion initiation, meaning *de novo* generation of infectivity by spontaneous conversion of PrP^{C} to PrP^{Sc} without template, and prion propagation, *i.e.* conversion of PrP^{C} to PrP^{Sc} in the presence of PrP^{Sc} template, are likely to involve two different molecular mechanisms, both remaining as yet unresolved. Although it has long been possible to maintain prion propagation continuously in animals (8) and in cell culture (9, 10), only recently have there been significant advances in reproducing both prion initiation and propagation *in vitro* (11, 12).

Elucidating the NMR structure of the recombinant prion protein produced in *Escherichia coli* has been instrumental in determining the structural effects of disease-linked amino acid changes (13, 14). The mature prion protein (residues 23–231) can be divided into an N-terminal (23–120) and a C-terminal domain (121–231) (13). Whereas the C terminus adopts a mainly α-helical globular conformation, the N terminus is largely disordered (14), although it may adopt a non-random conformation at physiological pH (15). The most prevalent missense mutations causing fCJD are localized in the C-terminal domain and clustered at the edges of α-helical structures. However, recombinant PrP carrying disease-linked amino acid substitutions is not thermodynamically destabilized (16), pointing to a disease mechanism more complex than mere misfolding.

The N-terminal domain contains four highly conserved copper binding octarepeats (ORs) of the sequence PHGGGWGQ (single letter

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[2] The abbreviations used are: CJD, Creutzfeldt-Jakob disease; fCJD, familial CJD; DLS, dynamic light scattering; GST, glutathione *S*-transferase; NHa, normal non-infected hamster brain; OR, octarepeat; PK, proteinase K; PrP, prion protein; *PRNP*, prion protein gene; ScHa, scrapie-infected hamster brain; SEC, size exclusion chromatography; BSA, bovine serum albumin; NTA, nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.

amino acid code; residues 60–91). These are flanked by one nonarepeat (residues 51–59; PQGGGTWGQ) and one partial repeat (residues 92–98; GGGTHNQ) that could bind copper as well (17). The OR domain binds copper in a cooperative manner at physiological pH and undergoes a distinct conformational change as a result, whereas copper affinity is abolished below pH 6 (18, 19).

The N-terminal domain of PrP, including the OR domain, is of little importance for prion propagation because removal of the N terminus from PrP^{Sc} by partial protease digestion does not significantly alter infectivity titers (20). Likewise, transgenic mice expressing PrP constructs with a deleted OR domain on the PrP knock-out background can still produce infectious prions, albeit with increased incubation times and reduced prion titers when inoculated with full-length prions (21–24). The redundancy of the OR domain for prion propagation stands in contrast to its genetic linkage to fCJD when the OR domain is expanded (25–27), indicating that the OR region does play a decisive role in prion initiation (28).

Our goal was to investigate the pathogenesis of fCJD by determining how OR domain expansion, being the result of an insertional mutation, starts the PrP misfolding pathway and ultimately leads to the formation of infectious prions. In a series of biochemical and biophysical experiments, we demonstrated how the OR domain mediates copper-dependent and -independent homomeric interactions between PrP molecules. OR domain expansion changes these properties in such a way that binding between OR domains is no longer fully reversible and binding to PrP^{Sc} instead of PrP^{C} is favored. Thus, by preferentially interacting with PrP^{Sc}, PrP^{C} with an expanded OR domain may have a higher likelihood of undergoing conversion, thereby facilitating development of fCJD.

EXPERIMENTAL PROCEDURES

Cloning

SyHaPrP-8OR, -10OR, and -16OR fragments were assembled from the following oligonucleotides: 1) phos-5′-GGCTGGGGGCAGCCCC-ATGGTGGT-3′, 2) phos-5′-CTGCCCCCAGCCACCACCATGGG-G-3′, 3) phos-5′-GGATCCGCGCGCGCGC-3′, 4) phos-5′-GGCTG-GGGGCAGTGATAAGAATTCGAGAGAGAGA-3′, 5) 5′-GCGCG-CGCGCGGATCCCCCCATGGTGGT-3′, and 6) TCTCTCTCTCG-AATTCTTATCA. First, we ligated oligonucleotides 2, 3, and 5 (10 μM each in 10 μl) using *Taq* ligase (New England Biolabs; 45 min, 45 °C). This ligation mix was then diluted 1/50 (mol/mol) in a mix of primers 1 and 2 (20 μM each, in 10 μl), followed by further ligation with *Taq* ligase (1 h, 40 °C). We then added primers 4 and 6 (250 nmol each) and ligated with T4 ligase (16 h, 16 °C). The resulting mixture was separated on 1.5% Tris borate-EDTA-agarose, and all fragments ≥200 bp were collected. The wild-type SyHaPrP-(23–98) and SyHaPrP-(52–98) fragments were amplified from pET-11a(SyHaPrP-(23–231)). Finally, all OR fragments were cloned into the pGEX-4T-3 expression vector (Amersham Biosciences) at BamHI/EcoRI. All cloned constructs were verified by sequencing on an ABI Prism (PerkinElmer).

Recombinant Protein Expression and Purification

Free GST (vector only), GST·HD20, GST·HD51, and GST·OR fusion proteins were expressed in BL21(λDE3) according to standard methods. Following lysozyme lysis, the suspension was brought to 50 mM Tris, pH 8, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 0.2% sarkosyl, cleared (20 min, 20.000 × *g*), and affinity purified on glutathione-Sepharose (Amersham Biosciences). After elution, all proteins were directly treated with iodoacetamide (50 mM, 30 min, room temperature) to block free Cys residues on the GST moiety. The GST·OR fusions were further purified on Zn^{2+}-nitrilotriacetic acid (NTA)-agarose (Novagen). All proteins were then extensively dialyzed against 10 mM KPO_4, pH 7.5, 0.1 mM EDTA. SDS-PAGE analysis confirmed that batches of all GST·OR fusion proteins were consistently purified to homogeneity and migrated at their expected molecular masses (Table 1).

Covalent Coupling of GST·OR Fusions to Sepharose and OR Peptides to BSA

Coupling to Sepharose—GST, GST·HD20, GST·HD51, and GST·OR proteins were covalently coupled to *N*-hydroxysulfosuccinimide-activated Sepharose (Amersham Biosciences) in 50 mM KPO_4, pH 7.5, 0.3% sarkosyl, 50 μM EDTA (2 h, room temperature) at a protein concentration of 0.5 mg/ml and a coupling density of 5 mg/ml.

Peptide Synthesis—Peptides corresponding to SyHaPrP-(55–67) (1OR) and SyHaPrP-(55–98) (4OR) were synthesized by the Biomedizinisches Forschungszentrum at the University of Düsseldorf.

Coupling to Bovine Serum Albumin—1OR and 4OR peptides were linked to succinimidyl-acetylthioacetate (Sigma) and then combined with BSA (Bio-Rad) derivatized with succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Molecular Probes) at a 1/10 (mol/mol) ratio in 50 mM $NaPO_4$, pH 8, 50 mM hydrazine (2 h, room temperature). SDS-PAGE analysis showed that BSA-1OR/4OR conjugates carried several OR peptides each (data not shown).

Pulldown of PrP^{C} and PrP^{Sc} from Brain Extracts with Immobilized GST·OR Fusions

Capture of PrP^{C} and PrP^{Sc}—Normal hamster (NHa) or scrapie-infected hamster (ScHa; 263 K strain) brain homogenates (20% w/v stock in 50 mM HEPES, pH 7.5, 100 KAc, 250 mM sucrose, 5 mM $MgCl_2$, 5× protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride) were diluted to 0.5% (NHa) or 1% (ScHa) in binding buffer, pH 7.5 (50 mM HEPES, 10 mM Tris, pH 7.5, 300 mM NaCl, 0.6% Nonidet P-40, 0.3% sarkosyl) or binding buffer, pH 5.5 (100 mM NaAc, pH 5.5, 300 mM NaCl, 0.6% Nonidet P-40, 0.3% sarkosyl), both containing 1× protease inhibitors and either 50–200 μM $CuSO_4$/$ZnSO_4$ or 5 mM EDTA, and then cleared (5 min, 10,000 × *g*). Sepharose beads coated with GST, GST·HD20, GST·HD51, or GST·OR (20 μl) were combined with 0.5 ml of NHa or 1 ml of ScHa extract and incubated overnight at 4 °C. Beads incubated with NHa extract were washed and then boiled in 2× SDS-PAGE sample buffer. Beads incubated with ScHa extract were first split, *i.e.* half was boiled directly, while the other half was digested with 20 μg/ml PK (Merck) for 1 h at 37 °C in binding buffer, pH 7.5, plus 5 mM EDTA (stopped with 5 mM phenylmethylsulfonyl fluoride) prior to boiling. Samples were run on 12.5% SDS-PAGE, and blots were developed with a PrP monoclonal antibody 3F4 (29).

Removal of PrP^{C} from PrP^{Sc}—Sepharose beads coated with GST·16OR (20 μl) were incubated with ScHa extract (buffer, pH 7.5, plus 5 mM EDTA) obtained from infected hamsters in the terminal stage (low PrP^{C}/PrP^{Sc} ratio) or 42 days after infection (high PrP^{C}/PrP^{Sc} ratio). Beads were washed and then eluted with 50 ml of 20 mM HEPES, pH 7.5, 1 mM EDTA, 0.25–1.5% SDS (10 min, room temperature). After collection of the eluate, beads were washed with a further 1 ml of SDS buffer and then boiled.

HaPrP Enzyme-linked Immunosorbent Assay—For calibration, a stock solution of 0.5 mg/ml recHaPrP-(23–231) (30) was freshly diluted to 50–0.5 ng/ml in 100 mM $NaHCO_3$, pH 8.3, 7 M guanidinium HCl (GuHCl buffer) and coated onto Maxisorp plates (Nunc) overnight at room temperature. After blocking, wells were probed with monoclonal antibody 3F4 in 50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% BSA, 0.1% Tween 20, 0.1% Nonidet P-40 (2 h, room temperature) and then with peroxidase-labeled anti-mouse IgG (Pierce) in the same buffer (1 h, room temperature). Plates were developed with TMB substrate

TABLE 1

Description and measured particle sizes of GST·OR fusion proteins

The synthetic 8OR, 10OR, and 16OR inserts contain multiples of the PHGGGWGQ repeat. Properties shown are calculated molecular mass and hydrodynamic diameter (D_H ± S.D., in nm) and corresponding molecular mass as measured by dynamic light scattering at pH 7.5 and 5.5.

Construct	Calculated molecular mass	Measured D_H and molecular mass at pH 7.5	Measured D_H and molecular mass at pH 5.5
	kDa	*nm/kDa*	*nm/kDa*
GST	26.2	5.4 ± 0.2/34 ± 4	
GST·SyHaPrP-(23–98) (4OR)	33.7	6.8 ± 0.2/59 ± 4	
GST·SyHaPrP-(52–98) (4OR)	30.7	6.0 ± 0.2/44 ± 4	
GST·8OR	32.5	10.6 ± 0.6/170 ± 25	
GST·10OR	34.1	15.8 ± 0.4/420 ± 30	7.8 ± 0.3/79 ± 8
GST·16OR	38.7	21.2 ± 0.6/840 ± 60	10.6 ± 0.6/170 ± 25

(Pharmingen; 15 min, room temperature) according to the manufacturer's protocol.

Quantification of GST·16OR-bound PrP^{Sc} by Sequential Pulldown—1 ml of 1% ScHa extract was sequentially incubated with two batches of 150 μg of Sepharose-linked GST·16OR (overnight, 4 °C). In parallel, we incubated ScHa homogenate (diluted), the pellet thereof (resuspended in 1 ml of binding buffer, pH 7.5), and extract without Sepharose beads. These samples, in parallel with the extract after GST·16OR pulldown, were digested with PK (20 μg/ml, 1 h at 37 °C), after which PrP^{Sc} was pelleted (1 h, 120,000 × *g*) and washed once with 1 ml of 100 mM $NaHCO_3$, pH 8.3. Pellets were then taken up in 200 μl of GuHCl buffer. Following pulldown, beads were washed with binding buffer, pH 7.5, and 100 mM $NaHCO_3$, pH 8.3, and then PK digested and subsequently extracted with 200 ml of GuHCl buffer. The PrP content of all GuHCl samples was determined by enzyme-linked immunosorbent assay as described above.

Size Exclusion Chromatography

Purified GST·4OR, GST·10OR, and GST·16OR (3 mg/ml) were fractionated on a HiPrep 16/60 Sephacryl S-200 HR column (Amersham Biosciences) at 0.5 ml/min in binding buffer, pH 7.5, plus 5 mM EDTA or in 20 mM NaAc, pH 5.5, 2 mM EDTA, 0.3% sarkosyl using a Biologic LP system (Bio-Rad). Calibration was done using size exclusion chromatography (SEC) standards (Bio-Rad). Fractions were analyzed on 4–20% SDS-PAGE gels (Bio-Rad), stained overnight with SYPRO Ruby (Bio-Rad).

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were performed on a DynaPro-MS/X machine (Protein Solutions). BSA, BSA-1OR, and BSA-4OR were diluted to 30–250 μg/ml in 50 mM HEPES, pH 7.5, 150 mM NaCl, supplemented with either 200 μM $CuSO_4$ or 2 mM EDTA. GST and GST·OR fusions were measured at dilutions of 100–1000 μg/ml in 50 mM HEPES, 5 mM EDTA, or in 100 mM NaAc, pH 5.5.

Scanning Force Microscopy

GST·OR fusions were deposited on a freshly cleaved mica surface in 5 mM HEPES/MES, pH 7.9/5.5, 3 mM KCl, 5.5 mM $MgCl_2$, air-dried, and analyzed as described (31).

Animal Inoculations

Syrian Gold hamsters (6–8 weeks old) were inoculated intracerebrally using a 24-gauge needle (four or five hamsters each group) with the following material. (A), starting material: 1 ml of 1% ScHa brain extract (263 K) in binding buffer, from which PrP^{Sc} was collected by ultracentrifugation (45 min, 100,000 × *g* in an Optima table ultracentrifuge (BeckmanCoulter)) and subsequently washed twice with 70% ethanol and twice with sterile PBS. (B), beads coated with GST·16OR (see above) that had been incubated overnight in ScHa extract produced as in (A) and then washed three times in binding buffer, twice with 70% ethanol, and twice with sterile PBS. (C), 1 ml 1% ScHa brain extract in binding buffer after pull down with GST·16OR prepared as described in (A). (D), as a negative control, GST·4OR beads were prepared as in (B). (E) As another negative control, GST beads were prepared as for (B). For (B), (D), and (E), we choose to inoculate the whole bead fraction in order to investigate all infectivity captured and to avoid manipulating infectivity by elution procedures. Animals were examined daily for standard neurological symptoms and were sacrificed because of animal protection aspects when severe clinical symptoms were observed. The animal experimentation protocol had been approved to Lothar Stitz.

RESULTS

The Wild-type Prion Protein OR Domain Is a Reversible, Copper-dependent Self-association Domain—First, we established the copper-dependent mode of the homomeric interactions between OR domains. Glutathione *S*-transferase (GST) fusion proteins, in which GST was linked N-terminal to SyHaPrP N-terminal fragments with different OR lengths (Table 1) were used to circumvent poor solubility of both wild-type and expanded OR domains when present as free polypeptides or within full-length PrP (data not shown). Because the octarepeat sequences of human and hamster PrP are identical, we considered these constructs to be valid models for investigating biochemical characteristics of the OR domain in human PrP. We covalently coupled GST alone, GST with four ORs (GST·4OR), and GST with sixteen consecutive ORs (GST·16OR) to Sepharose via amine linkage, thus ensuring that only the GST moiety was bound to the solid support. GST·16OR was used as a model protein for expanded OR domains as occurring in fCJD, where the maximum number of ORs reported so far is 14. When incubated with brain extract from normal, non-infected hamsters (NHa) in sarkosyl-containing buffer (0.3%), both the GST·4OR and GST·16OR captured PrP^C in the presence of copper ions at pH 7.5 with a half-maximal effect between 75 and 125 μM (Fig. 1*A*). However, unlike GST·4OR, GST·16OR still retained PrP^C even in the absence of copper ions (Fig. 1*B*), suggesting partial loss of copper-dependent reversibility for PrP binding. Under the conditions used here, the full N-terminal fragment PrP-(23–98) expressed as a fusion protein to GST (GST·SyHaPrP-(23–98)) showed essentially the same effect as GST·4OR (data not shown), demonstrating that the OR domain alone is sufficient for PrP^C binding. Experiments performed with zinc yielded the same results as copper over the same concentration range.

To establish whether OR domains could interact directly in solution and to analyze the critical OR length needed for such an interaction, we covalently linked synthetic 1OR (residues 55–67) and 4OR (residues 55–98) peptides via amine linkage to BSA and analyzed copper-dependent OR-OR interactions *in vitro* by DLS. In the absence of copper ions (2 mM EDTA), the hydrodynamic diameters (D_H) of BSA alone, BSA-1OR,

and BSA-4OR were 7.5 ± 0.4 nm (70 ± 10 kDa), indicating that all three conjugates were essentially monomeric. Adding copper ions (200 μM $CuSO_4$) caused BSA-4OR, but not BSA or BSA-1OR, to associate into large, heterogeneous particles (D_H ≥85 nm), indicating binding between BSA-4OR conjugates, each carrying several peptides. These results demonstrated that 4OR, but not 1OR, peptides directly self associate in the presence of copper ions, presumably because of the conformation-inducing effect of copper binding on the OR domain (19). We were unable to determine the effect of copper on the size distribution of GST·OR proteins by DLS as GST itself was no longer monodisperse in the presence of copper, thus prohibiting reliable data collection.

The Expanded OR Domain as in fCJD Leads to the Formation of Distinct Multimeric Complexes—When we examined GST·16OR by DLS in the absence of copper ions, we found that it was present as a monodisperse multimeric complex with a D_H of 21.2 ± 0.6 nm, corresponding to 850 ± 50 kDa at pH 7.5 (Table 1). Under the same conditions, GST alone and GST·4OR were measured to be essentially monomeric (Table 1). These findings demonstrate that OR domain expansion brings about new homomeric interactions that are copper independent and ordered in nature. Interestingly, GST·OR proteins with intermediate OR lengths also formed particles of intermediate size: GST·8OR and GST·10OR had diameters of 10.6 ± 0.6 nm (160 ± 30 kDa) and 15.8 ± 0.4 nm (420 ± 30 kDa), respectively, demonstrating a gradual effect of OR length on multimerization. Upon lowering the pH to 5.5, multimeric GST·16OR readily dissolved into lower molecular mass complexes with a D_H of 10.7 ± 0.6 nm (170 ± 30 kDa). Likewise, GST·10OR multimers converted to monomer- or dimer-like particles (Table 1), confirming that higher order multimerization by the expanded OR domain is a phenomenon that only occurs at physiological pH.

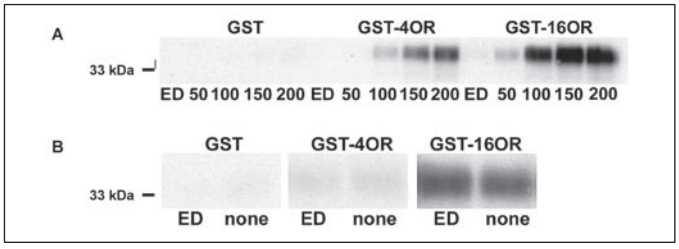

FIGURE 1. **The OR domain is a copper-dependent self-association domain.** *A*, Western blot of pulldown experiments with Sepharose-bound GST, GST·4OR, and GST·16OR from normal hamster extracts in the presence of copper. Per lane, pulldown from 0.5 ml of 0.5% NHa extract in binding buffer, pH 7.5, plus 5 mM EDTA (*ED*) or 50–200 μM $CuSO_4$ as indicated. GST·4OR and GST·16OR both pull down PrP^C in the presence of copper ions. *B*, longer exposure of film reveals that only GST·16OR also pulls down PrP^C in the absence of copper ions. *None*, no additives; *ED*, plus 5 mM EDTA.

Our DLS findings on multimerization of expanded OR domains were confirmed by scanning force microscopy and SEC (Fig. 2, *A* and *B*, respectively). Scanning force microscopy analysis demonstrated that, compared with GST·4OR, all (detergent-free) GST·10OR and GST·16OR multimers appeared as essentially homogenous, spherical particles and not as, for instance, fibrillar species (Fig. 2*A*). Indeed, purified GST·16OR did not bind thioflavin T, indicating that these multimers were not amyloid like (data not shown). Quantitative analysis of scanning force microscopy images showed that GST·16OR multimers had a diameter of 46.3 ± 9.8 nm (Fig. 2*A*); this apparent discrepancy with the multimer size determined by DLS (~21 nm) was most likely because of tip convolution effects. Furthermore, SEC analysis demonstrated that GST·16OR multimers, but not those of GST·10OR, were stable in 0.3% sarkosyl and that GST·16OR multimers converted to oligomers at pH 5.5 (Fig. 2*B*) in a manner that was consistent with our DLS measurements. Taken together, our results demonstrate that OR domains containing at least 8 repeats can form homogenous multimeric complexes of distinct size under physiologically relevant conditions, indicating that increasing the number of ORs favors the formation of stable homomeric complexes of PrP.

The Mutant Expanded, but Not Wild-type, OR Domain Binds PrP^{Sc}—We went on to investigate whether, in parallel to multimerization, wild-type and expanded OR domains differed in their interaction with PrP^{Sc},

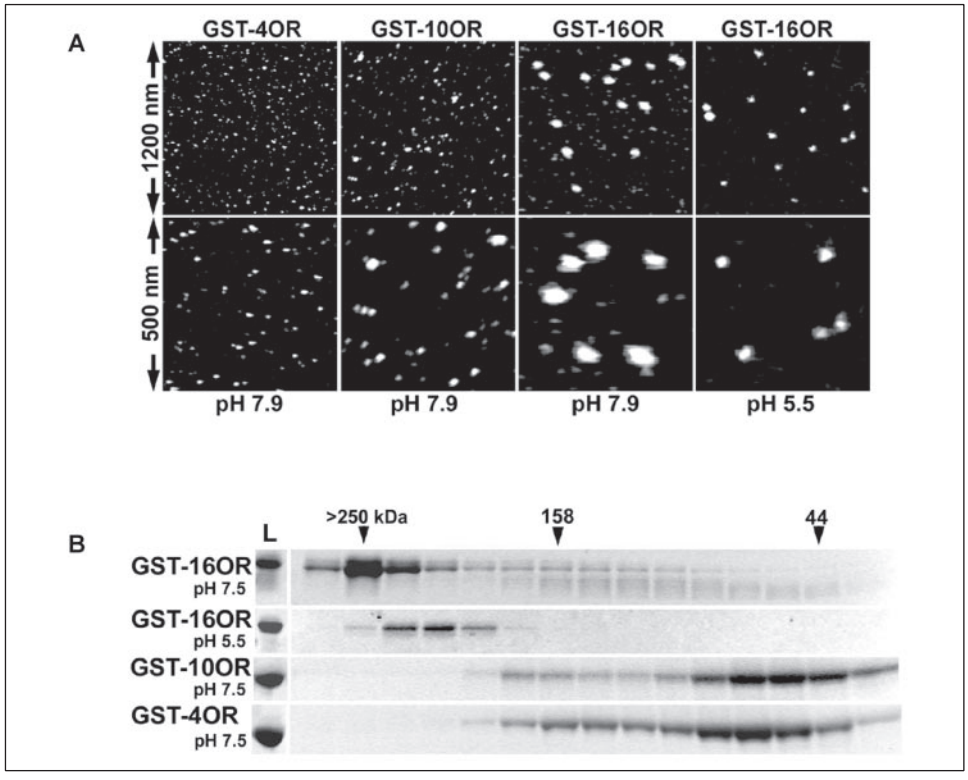

FIGURE 2. **GST expanded OR fusion proteins form distinct multimeric complexes.** *A*, scanning force microscopy analysis of GST·4OR, GST·10OR, and GST·16OR at pH 7.9 or 5.5 (as indicated). A gradual, pH-dependent multimerization from GST·4OR to GST·16OR is observed. *B*, protein stain (SYPRO Ruby; Bio-Rad; negative image) of fractions from SEC analysis of GST·4OR, GST·10OR, and GST·16OR (as indicated on *left side*, *lane L* shows starting material) on a HiPrep 16/60 Sephacryl S-200 HR column. Calibrated molecular mass standards are indicated in the top row. Whereas GST·4OR and GST·10OR are essentially monomeric, GST·16OR has a size of >250 kDa at pH 7.5 and between 170 and 200 kDa at pH 5.5.

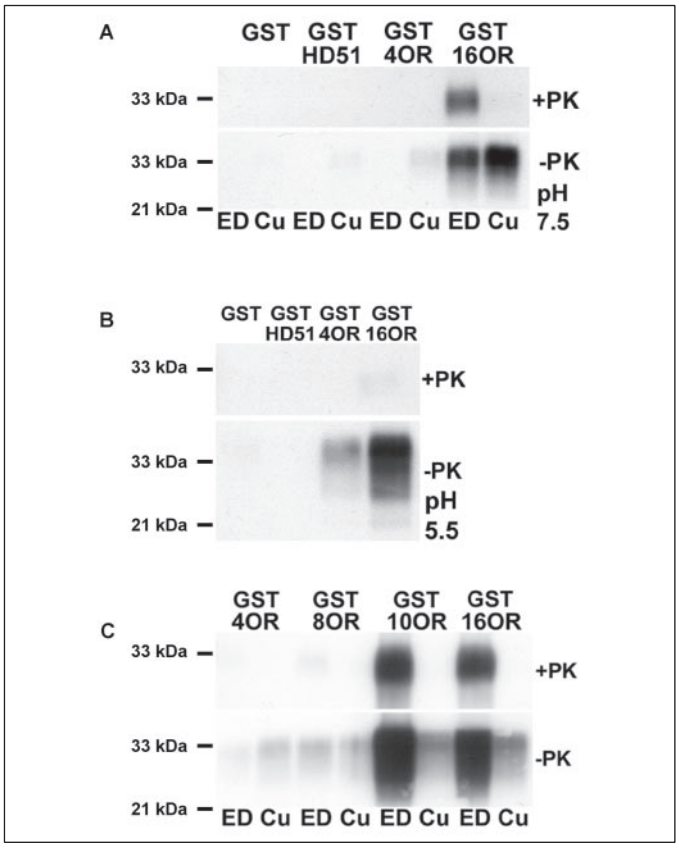

FIGURE 3. **An expanded OR domain of at least 10 consecutive repeats binds PrP^{Sc}.** *A*, Western blot of pulldown experiments with Sepharose-bound GST, GST·HD51, GST·4OR, and GST·16OR (as indicated) from ScHa extracts in the presence of 200 μM $CuSO_4$ (*Cu*) or 5 mM EDTA (*ED*) at pH 7.5. GST·16OR binds PK-resistant PrP^{Sc} in the presence of EDTA. No binding was observed by either construct at pH 5.5 (*B*). *C*, pulldown experiments using GST·OR proteins with 4, 8, 10, and 16 repeats (as indicated in top row) show that only GST·10OR or GST·16OR bind PrP^{Sc}. *A–C*, PK-digested samples (*upper panel*) and undigested samples (*lower panel*).

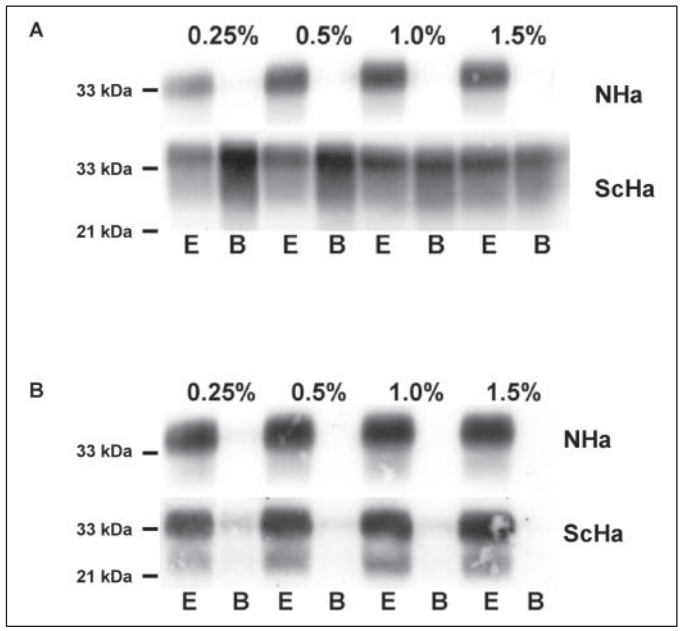

FIGURE 4. **The GST·16OR-PrP^{Sc} complex is SDS-resistant, whereas the GST·16OR-PrP^{C} complex is not.** Western blot of eluted (*E*) and bound PrP (*B*) after washing beads from pulldown experiments with Sepharose-bound GST·16OR from normal hamster (*NHa*) extract (*upper panel*) or scrapie-infected hamster (*ScHa*) extract (*lower panel*) with 0.25–1.5% SDS (as indicated on top). ScHa brains were from terminally ill hamsters (*A*) or asymptomatic hamsters at day 42 after inoculation (*B*). Whereas PrP^{C} is removed with ≥0.5% SDS (*upper panels*), only PrP from scrapie-infected hamsters (presumably PrP^{Sc}) remains bound to beads (*lower panel A*). SDS-resistant binding of PrP^{Sc} was also observed when only small amounts of PrP^{Sc} were present in the brains of asymptomatic inoculated hamsters (*lower panel B*).

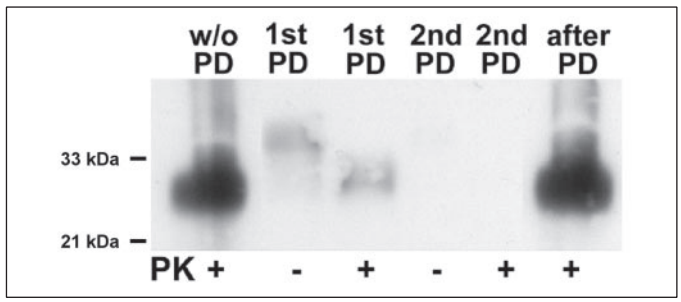

FIGURE 5. **GST·16OR binds a small fraction of total PrP^{Sc}.** Western blot of pulldown with Sepharose-bound GST·16OR from ScHa extracts in binding buffer, pH 7.5, plus 5 mM EDTA. Each lane corresponds to 0.5 ml of 1% ScHa extract. The starting material (*w/o PD*) and extract after pull down (*after PD*) were PK digested and ultracentrifuged to collect all PK-resistant PrP^{Sc}. Two sequential pulldowns with GST·16OR (*1st PD* and *2nd PD*) from the starting material were performed. Of each pulldown, half of the beads were PK digested (+*PK*) and the other half eluted directly by boiling in 2× SDS-PAGE sample buffer (−*PK*). The blot shows that only a fraction of PK-resistant PrP present in starting material was captured (compare *w/o PD* to *1st* +*PK*) and that the second pulldown did not yield additional PK-resistant PrP (compare *1st* to *2nd* +*PK*).

which could indicate that the expanded OR domain stabilizes this pathological conformation. When we incubated Sepharose-immobilized GST·OR fusion proteins with brain extract from ScHa in the presence of sarkosyl-containing buffer, we observed that only GST·16OR captured PrP^{Sc} at pH 7.5 in the absence of copper ions (Fig. 3, *A* and *B*), demonstrating selective interaction of the expanded OR domain with PrP^{Sc}. Adding copper or zinc (200 μM $CuSO_4$/$ZnSO_4$) or lowering the pH to 5.5 during incubation essentially abolished PrP^{Sc} binding. As controls, we verified that both GST·HD20 and GST·HD51, GST fusion proteins with the huntingtin exon-1 polypeptide containing a sequence of 20 or 51 glutamine residues, respectively (32), did not bind PrP^{Sc} (Fig. 3*A* for GST·HD51, GST·HD20 data not shown), thereby ruling out nonspecific interactions with PrP^{Sc}. At pH 5.5, GST·16OR did not bind protease-resistant PrP^{Sc} (Fig. 3*B*), but both the GST·4OR and, especially, GST·16OR did bind PK-sensitive PrP, possibly PrP^{C}.

A Threshold of 10 OR in the Expanded OR Domain Establishes a PrP^{Sc} Binding Site—To determine how many consecutive ORs were needed for the emergence of the PrP^{Sc} binding site in the expanded OR domain, we performed pulldown experiments from ScHa brain extracts with GST·OR proteins of different OR lengths. We observed a clear threshold effect, namely a complete switch from no to full PrP^{Sc} binding between eight and ten ORs (Fig. 3*C*). Remarkably, ten ORs has previously been reported to be the minimum number of OR to be required for transmissibility in fCJD with expanded OR (28). As with GST·16OR, the presence of copper ions inhibited binding of PrP^{Sc} to GST·10OR. Having shown by SEC analysis that GST·10OR was not multimeric under binding conditions used here, we conclude that it is an intrinsic conformational change of the expanded OR domain that creates a PrP^{Sc} binding site rather than its multimerization.

Resistance to Denaturing Buffer Conditions Demonstrates Tight Binding between PrP^{Sc} and the Mutant, Expanded OR Domain—Because GST·16OR bound both PrP^{C} and PrP^{Sc} at physiological pH and in the absence of copper ions, both forms were invariably retained during a pulldown experiment from ScHa extract (Fig. 3*A*). To investigate differences between PrP^{C} and PrP^{Sc} binding to GST·16OR and to define conditions where GST·16OR could select between the two PrP isoforms, we tested a range of washing buffers for their ability to remove PrP^{C} while

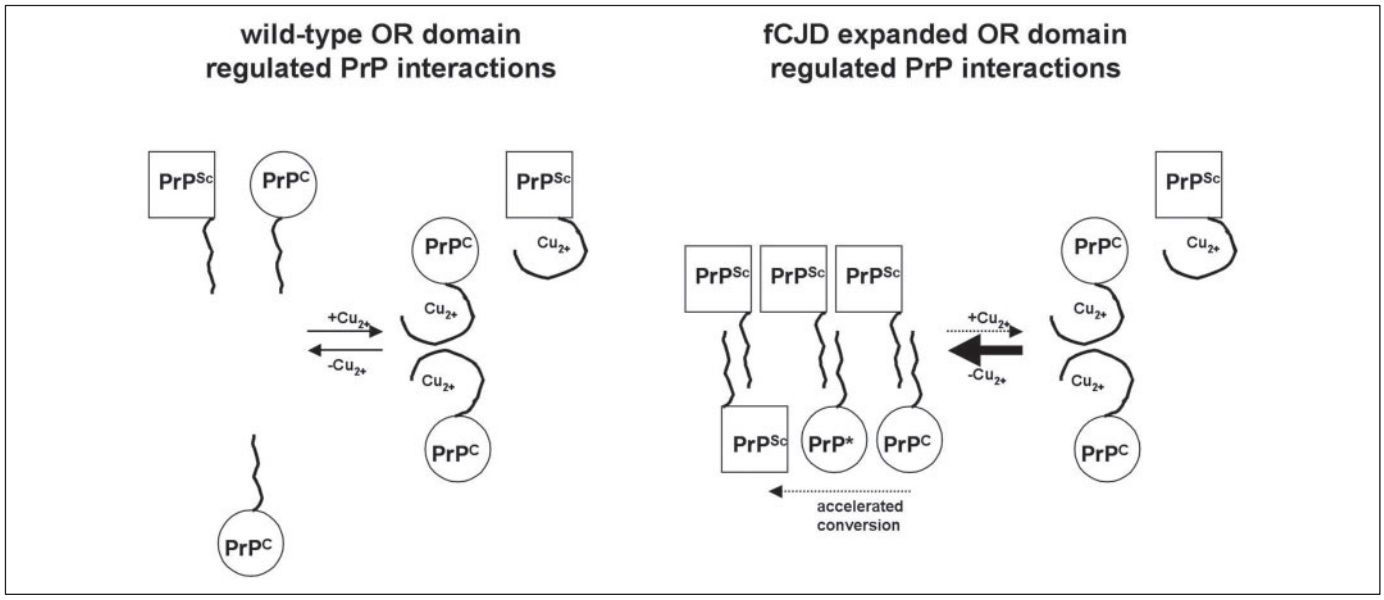

FIGURE 6. **Schematic drawing of how expanded OR domains change homomeric interactions of the N-terminal domain of PrP to favor conversion to PrP^{Sc} in fCJD.** PrP^{C} conformation in *circles*, PrP^{Sc} conformation in *squares*. N-terminal domain depicted as non-coordinated (*loose tails*) or copper coordinated (*round tails*). A multimeric cluster of prion proteins carrying expanded octarepeat domains may therefore capture and irreversibly incorporate spontaneously arising short-lived PrP^{Sc}-like conformers and thereby provide a matrix for their conversion.

retaining PrP^{Sc}. We found that PrP^{C} could be removed by washing with at least 0.5% SDS, while leaving PrP^{Sc} bound to GST·16OR (Fig. 4*A*). Attempts at achieving the same kind of separation using sarkosyl (5%), urea (10 M), high ionic strength (1 M NaCl), low pH (10% acetic acid), or copper ions (up to 200 μM) were unsuccessful (data not shown). By means of the SDS washing technique, we were able to detect a small amount of PrP^{Sc} in ScHa extract even at a high PrP^{C}/PrP^{Sc} ratio, namely in brain homogenates from asymptomatic scrapie-infected Syrian hamsters (culled at day 42 after inoculation of a 60-day incubation period; Fig. 4*B*). These results clearly show how effectively PrP^{Sc} is captured by the expanded OR domain even when relatively low levels of PrP^{Sc} are present in early stages of disease.

The Expanded OR Domain Recognizes a Distinct Subpopulation of PrP^{Sc} Molecules—To investigate how efficient recruitment of PrP^{Sc} by expanded octarepeats was, we quantified the amount of PrP^{Sc} that we could pull down from ScHa brain extracts. Surprisingly, only a small fraction of the total amount of available PK-resistant PrP^{Sc} was pulled down (Fig. 5). When the supernatant of the first pulldown was again probed with GST·16OR, no additional PK-resistant PrP^{Sc} was bound, indicating that the first round had depleted the brain homogenate of a particular PrP^{Sc} species present in the "total" PrP^{Sc} population under the experimental conditions used here (Fig. 5). Quantification of the pulled down fraction by enzyme-linked immunosorbent assay demonstrated that this GST·16OR-specific PrP^{Sc} species made up ~4% of the total amount of PK-resistant PrP^{Sc} present in the extract that itself contained 70% of total PK-resistant PrP^{Sc} in ScHa brain. The PrP^{Sc} species pulled down consisted of full-length PrP^{Sc} that was primarily double glycosylated, although other PrP glycoforms were also pulled down (see Figs. 3, 4, and 5). On undigested pulled down samples, no PrP fragments could be detected, indicating that the subpopulation of PrP^{Sc} pulled down consisted mostly of full-length PrP. When that material was protease digested, a shift in PrP immunoreactivity with an electrophoretic mobility similar to that of the starting material was observed (see Fig. 5) and there was no decrease in signal intensity, demonstrating that all pulled down material consisted of protease-resistant full-length PrP^{Sc}. Thus, the pulled down PrP^{Sc} fraction probably corresponded to a particular conformation within a seemingly heterogenous population of PrP^{Sc}. These data parallel those under "Results" (Figs. 2 and 3) where we found that an OR length-dependent conformational change in the expanded OR domain rather than multimerization of GST·OR molecules created the novel PrP^{Sc} binding site (Figs. 2 and 3).

GST·16OR pulled down material inoculated into Syrian Gold hamsters demonstrated infectivity with an average time to death of 89 ± 7 days (4 of 4 hamsters dead, compared with 77 ± 5 days for starting material or material after GST·16OR extraction). Because incubation time of the GST·16OR-captured infectivity was significantly shorter than that of negative controls (GST·4OR, 98 ± 12 days to death (Student's *t*-test $p < 0.001$); GST alone, 107 ± 19 days to death (Student's *t*-test $p < 0.001$)), these experiments indicate that the PrP^{Sc} species pulled down was associated with infectivity. The presence of infectivity in negative controls was unavoidable because the beads could not be washed harshly enough without interfering with prion infectivity itself. Our results thus provide evidence for the heterogeneity of the PrP^{Sc} population. To our knowledge, GST·16OR is the first ligand described that specifically targets an infectious subpopulation of PrP^{Sc}.

DISCUSSION

Expansion of the OR domain profoundly changes the reversible, homomeric, and copper-dependent interactions that are mediated by the N-terminal OR-containing domain of PrP. Our studies identified three new features that arise from OR domain expansion, namely partial loss of reversibility of copper-dependent interaction, gain of a PrP^{Sc} binding site, and gradual multimerization ability. Although our data do not reveal how the expanded OR directs protein misfolding of PrP^{C} to PrP^{Sc}, our results permit us to propose a model for the events preceding prion conversion in fCJD with insertional mutations (see Fig. 6). Our model addresses interactions between mutant PrP molecules and how these could favor prion conversion but does not relate to any intrinsic conformational shift toward PrP^{Sc} that might be brought about by OR domain expansion. In the presence of copper and at physiological pH, the OR domain with wild-type 4 ORs undergoes transient, reversible homomeric interactions with PrP^{C} but not with PrP^{Sc} (see also Fig. 3).

This interaction mode is based on a conformational change in the OR domain that is induced by copper binding (19). The expanded OR domain behaves in the same way, with the exception that it does not fully release PrP^{C} upon copper depletion. In the absence of copper, the wild-type OR domain loses all affinity for PrP^{C}, whereas the expanded domain now tightly binds PrP^{Sc} and forms distinct multimers. When these two properties act either simultaneously or consecutively, it is likely that incorporating PrP^{Sc} or transient PrP^{Sc}-like conformers into a multimeric complex forms a nucleus for further PrP^{Sc} formation by favoring conversion. Our findings are paralleled in the prion-like (PSI+) determinant of yeast where a similar oligopeptide repeat PQGGYQQYN in Sup35 stabilizes intermolecular prion interactions and can be functionally replaced by the mammalian octarepeat peptides (33, 34).

The reversible, copper-dependent interactions of the wild-type four-OR repeat domain makes us think of a "copper switch." Only four Ors, but not one single OR peptide, constitute a copper switch, suggesting that the copper-induced conformational change of the OR domain as a whole rather than copper coordination alone is responsible for self association, in a manner similar to what has been reported by Viles *et al.* (19). Of note, it was found that PrP with nine extra ORs recombinantly expressed in cells did not undergo copper-induced endocytosis, whereas wild-type PrP did (35). This observation is consistent with a loss-of-function phenotype and with our finding that OR domain expansion interferes with the reversibility of the wild-type copper switch that might be crucial for this type of endocytosis. The ultimate purpose of reversible interactions of PrP with itself or other molecules is unknown, but from our results it is clear that these interactions are imbalanced when the OR domain is expanded by insertional mutations (see Fig. 6). Physiologically relevant reversible interactions of the OR domain would explain why the OR domain has been highly conserved during evolution by selecting against dysfunctional OR domains that contain more (or less) than the optimal four consecutive repeats.

Our findings can directly be related to clinical and neuropathological data from patients with fCJD with insertional mutations, thus offering a novel and intriguing mechanistic explanation for these phenotypes. An increased number of OR in fCJD cases decreases the age of onset of disease and duration of disease (3, 36). Moreover, it has been reported that brain tissue from fCJD patients carrying OR insertional mutations varies in infectivity, with the more expanded OR domains transmitting disease more efficiently (28, 37). These clinical phenotypes are paralleled by our results that show how multimerization progresses with increasing OR length and how PrP^{Sc} recruitment only occurs effectively with an OR length >10. Consequently, the combination of PrP molecules carrying an expanded OR domain together with PrP^{Sc} or PrP^{Sc}-like conformers in one stable multimeric complex might facilitate further conversion to such an extent that the disease process is set in motion spontaneously. Our *in vitro* data are consistent with earlier experiments in which SyHaPrP with different OR lengths was transiently expressed recombinantly in cells. There, with insertional mutations at a threshold of at least seven OR, PrP became increasingly aggregated and developed a weak protease resistance (38).

Recapitulating our observations on the multimerization and PrP^{Sc} binding behavior of expanded OR domains, we would like to stress their specific nature and thereby their relevance to disease. First of all, the homogenous nature and strict pH dependence of GST·16OR multimers point to a degree of internal order and regular subunit structure that sets them apart from "random" or nonspecific aggregates. Taking this into account, our next three observations argue for a specific interaction between the expanded OR domain and PrP^{Sc}. First, there is a complete switch from no to full PrP^{Sc} binding upon going from eight to ten ORs. Such an effect is unlikely to reflect nonspecific binding, as that is expected to show a more gradual increase. Second, GST·16OR exclusively binds a small and depletable subfraction of the overall amount of PK-resistant PrP^{Sc}. Such binding behavior is equally unlikely to stem from a nonspecific interaction between multimeric GST·16OR and a "sticky" target. In fact, such selectivity has not been reported for any other PrP^{Sc}-specific ligand (39, 40). Third, both GST·HD20 and GST·HD51, GST fused to mammalian polyglutamine-containing protein fragments with low and high aggregate/amyloid-forming propensity (32), respectively, lack all PrP^{Sc} binding ability. The PrP^{Sc} fraction purified with GST·16OR retained infectivity, thus excluding the possibility that a biologically irrelevant fraction of protease-resistant material had been isolated. The fact that the complex between GST·16OR and PrP^{Sc} is resistant to harsh or denaturing conditions may be because the GST·16OR multimer offers a very large and multifaceted binding surface for multimeric PrP^{Sc}, causing it to become kinetically trapped, especially when several GST·16OR multimers participate in binding.

Whether missense mutations causing other forms of fCJD could also act by recruitment of PrP^{Sc} and subsequent conversion enhancement is unclear. Nevertheless, the report that PrP with expanded OR domains, but not other missense mutations, converts PrP^{C} from non-mutant alleles (41) suggests that such a mechanism may be unique to fCJD with insertional mutations. Until now, animal models have failed to accurately mimic genetic prion disease. For example, a transgenic mouse strain (Tg(PG14)) expressing a nine-OR insertion homologue within epitope-tagged MoPrP failed to generate spontaneous infectivity even though these mice developed spontaneous neurodegenerative disease and were susceptible to mouse-adapted prions (7). The inability to mimic fCJD in a transgenic mouse model may be because of molecular differences in host factors essential for prion propagation and/or require mutated PrP to be expressed within the human amino acid sequence and eventually within the human genetic background.

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